Expression of survivin and cortactin in colorectal adenocarcinoma: Association with clinicopathological parameters

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Abstract. *Objective*: Survivin and cortactin are factors that promote tumor progression. We tested the hypothesis that survivin and cortactin expressions correlate with the clinico-pathological parameters of colorectal adenocarcinomas and survival time.
Methods: Immunohistochemical analysis of survivin and cortactin were performed using tissue microarrays of 119 specimens from 18 well, 50 moderately, and 27 poorly differentiated colorectal adenocarcinomas and 24 colorectal adenomas with dysplasia. As control, 10 specimens of normal colorectal epithelia were included.
Results: The percentage of cells immunostained and the immunostaining scores for survivin and cortactin were all significantly higher in well-, moderately, and poorly differentiated colorectal adenocarcinomas than in normal colorectal epithelia. The survivin immunostaining score was significantly correlated with T, M, and AJCC/TNM stages ($p < 0.05$). For cortactin, the score was significantly correlated with T and M stages ($p < 0.05$). Higher survivin immunostaining score was associated with higher mortality.
Conclusions: Higher expression of survivin and cortactin correlates significantly with tumor stages and shorter survival time. Survivin and cortactin may be good biomarkers of aggressiveness of colorectal adenocarcinomas. Our findings require validation in independent cohorts and these data support the potential targeting of survivin and cortactin for the development of novel therapeutic strategies.

Keywords: Adenocarcinoma, colorectal, survivin, cortactin, immunostaining scores

1. Introduction

The most common histological type of primary colon cancer is colorectal adenocarcinoma, accounting for 8.5% of all new malignancies [1]. Histopathological factors, such as differentiation grade, the depth of tumor invasion, and lymph node metastasis are associat-

ed with tumor prognosis [2–4]. Identification of mechanisms underlying tumor cell invasion may help direct development of new therapies that can arrest local invasion and metastatic spread of disease.

Survivin is a 16.3 kDa (142-amino-acid) protein that belongs to the inhibitor of apoptosis protein (IAP) family [5]. Many embryonic tissues and most malignant cells have significantly increased survivin expression [6]. Survivin affects cell proliferation [7], angiogenesis [8–10], and inhibition of apoptosis [6], but the exact mechanisms are still unclear [11] even though a recent study of colorectal adenocarcinoma showed

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survivin over-expression was related to tumor invasiveness, proliferation, and differentiation [13].

Cortactin is an actin-binding protein that activates the Arp2/3 complex to regulate the actin cytoskeleton [13] and inhibits debranching of dendritic actin networks [14]. The gene responsible for cortactin expression is on chromosome 11q13 and is frequently amplified in some human cancers, such as breast, head/neck carcinomas, and gastric adenocarcinoma [15–17]. Remodeling of the actin cytoskeleton has effects on cell migration, motility, and adhesion, as well as on tumor invasion and metastasis [13]. In some studies, the amplification of 11q13 and overexpression of cortactin correlate with poor prognosis for patients with lymph node metastasis [16–18]. However, the relationship between cortactin expression and clinicopathological parameters of colorectal adenocarcinoma is also unclear.

In this study, we tested the hypothesis that higher survivin and cortactin immunostaining scores in colorectal adenocarcinoma patients associate with advanced cancer stages and with decreased survival time.

2. Materials and methods

We selected 24 colorectal adenoma cases, and 95 cases of primary colorectal adenocarcinoma, including 18 well differentiated (glandular structure > 95%), 50 moderately differentiated (glandular structure 50%–95%), and 27 poorly differentiated adenocarcinomas (glandular structure < 50%). The histopathological differentiation of colorectal adenocarcinoma was determined according to the WHO criteria for tumor classification [19]. The 24 adenoma cases were from colonoscopic biopsy specimen and 10 normal samples were taken at least 10 cm from the specimens of colorectal adenocarcinoma.

Paraffin-embedded tumor tissue blocks were obtained, one core was taken from a selected area of each block, and tissue microarray slides were constructed according to a previously published method [20]. Each representative core in the tissue microarray slide was 1.5 mm in diameter. The tissue microarray slide and slides of the original paraffin-embedded specimens stained uniformly with H&E. Each pathological diagnosis in these cases was reviewed by at least two experienced pathologists who were blinded to each other's evaluation. If they disagreed, a third pathologist was consulted.

All tumors were pathologically staged according to the 2002 American Joint Committee on Cancer (AJCC/TNM) staging system. Normal colonic tissues were obtained from 10 cases and were taken at least 10 cm from the primary neoplasm. In no case was radiation or chemotherapy given before surgery.

2.1. Immunohistochemistry

Tissue microarray sections were de-waxed in xylene, rehydrated in alcohol, and immersed in 3% hydrogen peroxide for 5 minutes to suppress endogenous peroxidase activity. Antigen retrieval was performed by heating (100°C) each section for 30 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0). After 3 rinses (each for 5 minutes in phosphate buffered saline [PBS]), sections were incubated for 1 hour at room temperature with a monoclonal mouse anti-human survivin (clone 91630) antibody (1:100, R&D Systems, Wiesbaden, Germany) and a polyclonal rabbit anti-human cortactin (clone H-191) antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS. After 3 washes (each for 5 minutes in PBS), sections were incubated with biotin-labeled secondary immunoglobulin (1:100, DAKO, Glostrup, Denmark) for 1 hour at room temperature. After 3 additional washes, peroxidase activity was developed with diaminobenzidine (DAB; DAKO, Glostrup, Denmark) at room temperature. For assessment of survivin and cortactin immunostaining scores, the intensity of cytoplasmic and nuclear immunostaining was scored on a scale of 0 (no staining) to 4 (strongest intensity), and the percentage of cells with stained cytoplasm or nucleus was estimated at each intensity. The percentage of cells (from 0 to 100) was multiplied by the corresponding immunostaining intensity (from 0 to 4) to obtain immunostaining scores ranging from 0 to 400. The immunostaining scores are uniform distribution and continuous variable.

2.2. Statistical analysis

All results are expressed as mean ± standard error of the mean (S.E.M.). The immunostaining scores for survivin and cortactin in colorectal tubular adenomas and adenocarcinomas were compared with the score in normal colonic epithelia. Statistical analysis was performed using the Student t-test between groups and a p-value of less than 0.05 was considered to be statistically significant. SigmaState software (Jandel Scientific, San Rafael, CA, USA) was used to perform linear regression testing to analyze the relationship between survivin/cortactin expressions and clinicopathological parameters. For multivariate modeling, SAS Proc MI
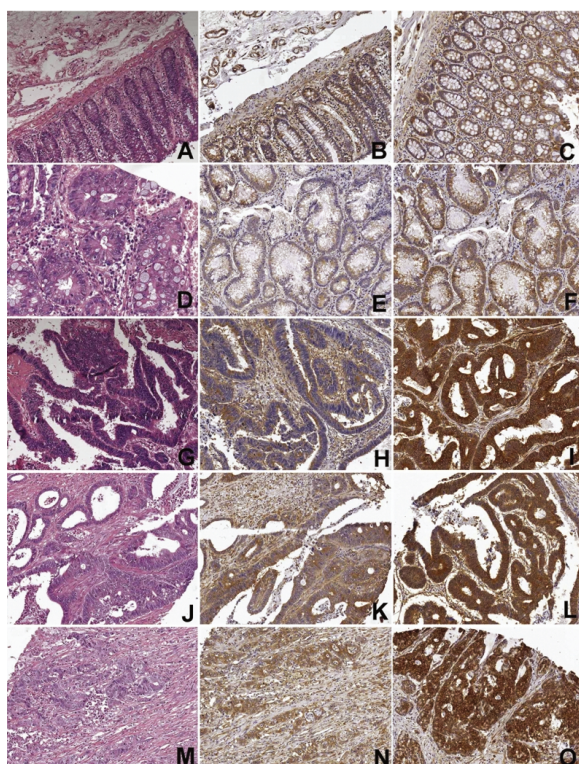
Fig. 1. Hematoxylin and eosin staining of normal colorectal epithelia (A), colorectal tubular adenoma (D), well differentiated (G), moderately differentiated (J), and poorly differentiated (M) colorectal adenocarcinomas; immunohistochemical analysis of survivin in normal colorectal epithelia (B), colorectal tubular adenoma (E), well differentiated (H), moderately differentiated (K), and poorly differentiated (N) colorectal adenocarcinomas; and immunohistochemical analysis of cortactin in normal colorectal epithelia (C), colorectal tubular adenoma (F), well differentiated (I), moderately differentiated (L), and poorly differentiated (O) colorectal adenocarcinomas.

Table 1
Clinicopathological characteristics of 94 patients with colorectum adenocarcinoma

Variable	n
Sex	
Male	46
Female	48
Histopathological differentiation	
Well-differentiated	18
Moderately-differentiated	50
Poorly-differentiated	26
TNM classification	
T1	2
T2	17
T3	58
T4	17
N0	53
N1	24
N2	17
M0	72
M1	22
AJCC stage	
I	16
II	31
III	25
IV	22

AJCC: American Joint Committee on Cancer.

(SAS Institute, Cary, NC) was used to input data. In addition, survival time of subjects was calculated from the date of surgery to the date of death. Ninety-five patients in the study received 5-year follow up; subjects were divided into two groups (high and low) for both cortactin and survivin immunostaining scores in order to compare survival times. There were 46 cases with higher survivin expression (score > 180) and 49 cases with low survivin expression (score < 180) and 48 cases with higher cortactin expression (score > 275) and 47 cases with lower cortactin expression (score < 275). The endpoint for this study is 5 years and crude survival was analysized. Statistical analysis of survival time was done using the Kaplan–Meier survival test. The number of events over the 5 year period were 45 patients died in cortactin score > 275, and 43 patients died in score < 275; and were 43 patients died in survivin score > 180, and 40 patients died in score < 180.

3. Results

The clinicopathological characteristics of 94 patients with colorectum adenocarcinoma was demonstrated in Table 1. There were 18 cases of well differentiated, 50 cases of moderately differentiated, and 26 cases of poorly differentiated cases.

3.1. Survivin and cortactin expression in colorectal adenocarcinoma

The staining intensity and percentage of cells immunostained for survivin in colon adenocarcinoma are shown in Table 2 and Fig. 1 (panels H, K, and N). The cytoplasmic survivin immunostaining scores were all significantly higher in well-, moderately, and poorly differentiated (the mean and standard error of mean were 166.7 ± 23.3, 191.9 ± 11.1, and 180.2 ± 48.4, respectively) adenocarcinomas than in normal colon epithelia (74.0 ± 14.4). There were low expression of survivin in the nucleus of tumor or normal epithelia and no significant difference was reached (Table 2).

The staining intensity and percentage of cells immunostained for cortactin in colon adenocarcinoma are

Table 2
Survivin immunostaining scores in colorectal adenocarcinoma

	Tumor		
	Intensity	% Staining	Total score
Cytoplasmic staining			
Tubular adenoma ($n = 24$)	1.7 ± 0.2*	41.6 ± 11.6	76.6 ± 14.3
Well differentiated ($n = 18$)	2.2 ± 0.2	66.7 ± 6.8*	166.7 ± 23.3*
Moderately differentiated ($n = 50$)	2.6 ± 0.1	72.0 ± 2.9*	191.9 ± 11.1*
Poorly differentiated ($n = 27$)	2.2 ± 0.4	78.5 ± 14.9*	180.2 ± 48.4*
Normal colon epithelia ($n = 10$)	2.3 ± 0.3	32.0 ± 5.0	74.0 ± 14.4
Nuclear staining			
Tubular adenoma (n =24)	0.2 ± 0.5	5 ± 3	1.2 ± 1.4
Well differentiated ($n = 18$)	0.3 ± 0.4	6 ± 5	1.8 ± 2.2
Moderately differentiated ($n = 50$)	0.4 ± 0.6	4 ± 6	1.6 ± 3.5
Poorly differentiated ($n = 27$)	0.3 ± 0.5	3 ± 6	0.9 ± 3.2
Normal colon epithelia ($n = 10$)	0.5 ± 0.4	5 ± 4	2.5 ± 1.5

Data are means ± standard error of the mean (SEM) of immunostaining score for cytoplasmic and nuclear staining of survivin in colorectal adenocarcinoma.
*Indicates significant difference in survivin expression between tumor and normal colorectal epithelia ($p < 0.05$).

Table 3
Cortactin immunostaining scores in colorectal adenocarcinoma

	Tumor		
	Intensity	% Staining	Total score
Cytoplasmic staining			
Tubular adenoma ($n = 24$)	2.5 ± 0.2	72.6 ± 6.4*	178.2 ± 14.4*
Well differentiated ($n = 18$)	3.2 ± 0.2	80.0 ± 6.8*	274.7 ± 26.2*
Moderately differentiated ($n = 50$)	3.1 ± 0.1*	84.8 ± 2.8*	266.1 ± 10.9*
Poorly differentiated ($n = 27$)	2.9 ± 0.4	87.8 ± 14.1*	266.9 ± 54.9*
Normal colon epithelia ($n = 10$)	2.6 ± 0.2	32.0 ± 5.3	86.0 ± 18.1
Nuclear staining			
Tubular adenoma ($n = 24$)	2.3 ± 0.8	29.6 ± 21.9	78.3 ± 66.6
Well differentiated ($n = 18$)	2.7 ± 1.0	44.4 ± 28.3	137.5 ± 92.7*
Moderately differentiated ($n = 50$)	2.9 ± 0.5*	52.8 ± 19.8*	161.9 ± 77.3*
Poorly differentiated ($n = 27$)	3.0 ± 0.7*	58 ± 24.8*	186.2 ± 93.0*
Normal colon epithelia ($n = 10$)	2.2 ± 1.1	29 ± 22.6	81 ± 81.8

Data are means ± standard error of the mean (SEM) of immunostaining score for cortactin in colorectal adenocarcinoma.
*Indicates significant difference in cortactin expression between tumor and normal colorectal epithelia ($p < 0.05$).

listed in Table 3 and Fig. 1 (panels I, L, and O). The cytoplasmic and nuclear cortactin immunostaining scores were all significantly higher in well-, moderately, and poorly differentiated adenocarcinomas than in normal colon epithelia.

3.2. Survivin and cortactin expressions in colorectal tubular adenoma

The staining intensity and percentage of cells immunostained for survivin and cortactin in colorectal adenoma are listed in Tables 2, 3, and Fig. 1 (panels E and F). Survivin immunostaining scores were similar in tubular adenoma (76.6 ± 14.3) and normal colon epithelia (74.0 ± 14.4). However, the cortactin immunostaining scores were significantly higher in tubular adenoma (178.2 ± 14.4 *versus* 86.0 ± 18.1 for colon epithelia).

3.3. The expressions of survivin and cortactin correlate with clinicopathological parameters

Linear regression testing was performed to analyze the relationship between survivin immunostaining score and clinical AJCC/TNM stages (Fig. 2) and between cortactin immunostaining score and AJCC/TNM stages (Fig. 3). The survivin immunostaining score correlated significantly with the T, M, and AJCC/TNM stages ($p < 0.05$) but not with N stage, while the cortactin immunostaining score correlated significant-
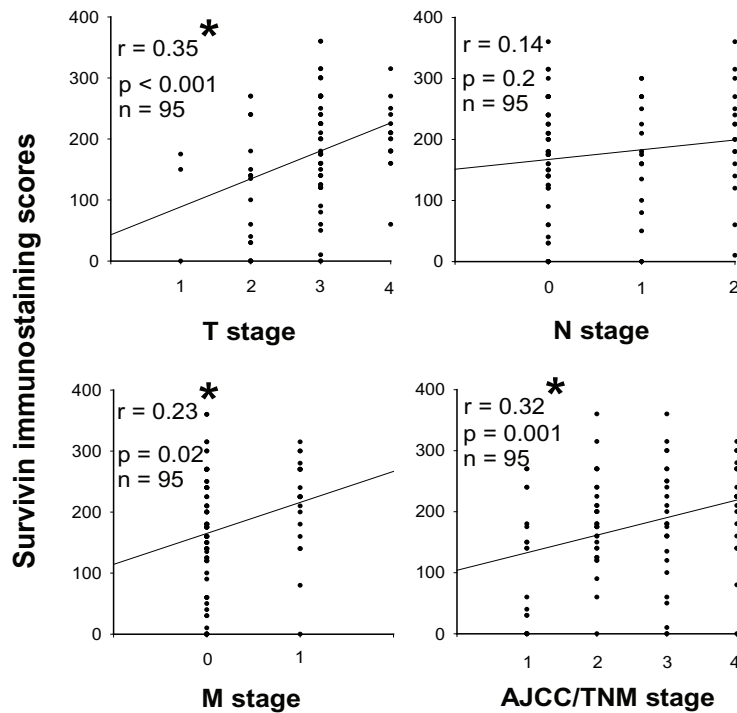
Fig. 2. Correlation of surviving immunostaining score and clinicopathological parameters in colorectal adenocarcinoma.
*Indicates statistical significance of Pearson's product moment correlation ($p < 0.05$).

ly with T and M stages ($p < 0.05$) but not with N or AJCC/TNM stages.

3.4. Correlation between survivin and cortactin expression

The correlation between survivin immunostaining scores and cortactin immunostaining scores is shown in Fig. 4. Significantly higher survivin immunostaining score was associated with elevated cortactin immunostaining score in colorectal adenocarcinoma.

3.5. Univariate and Multivariate analysis of survival time

In univariate analysis, we divided 95 patients with colorectal adenocarcinoma and 5–year follow up into two groups on the basis of survivin and cortactin immunoscores, respectively. Higher (immunostaining score $\geq$180) and lower (immunostaining score < 180) survivin expression occurred in 48 and 47 specimens, respectively, and higher (immunostaining score $\geq$ 275) and lower (immunostaining score < 275) cortactin expression occurred in 48 and 47 specimens, respectively. Higher survivin score (Fig. 5) but not higher cortactin

score (Fig. 6) was significantly associated with lower survival rate. The high survivin score (score $\geqslant$ 180), male patient, poor differentiation of tumor, large tumor size, positive for lymph node metastasis, and positive for distant metastasis, and higher TNM stage were predictive of inferior survival time (Table 4).

In a multivariate analysis survival analysis, the high survivin score (score $\geqslant$ 180), male patient, poor differentiation of tumor, large tumor size, positive for lymph node metastasis, and positive for distant metastasis were predictive of inferior survival time (Table 5).

4. Discussion

Our results demonstrated that survivin is a good biomarker in predicting clinical outcome, malignant transformation, and tumor progression in patients with colorectal adenocarcinoma.

In our study, all tumor tissues were placed in a single tissue-array slide. The tissue microarray technique is a powerful tool for simultaneous histological and immuno-histochemical evaluation of tumors [21]. Previous studies measuring immunohistochemical intensity of individual cases were limited because of the vari-
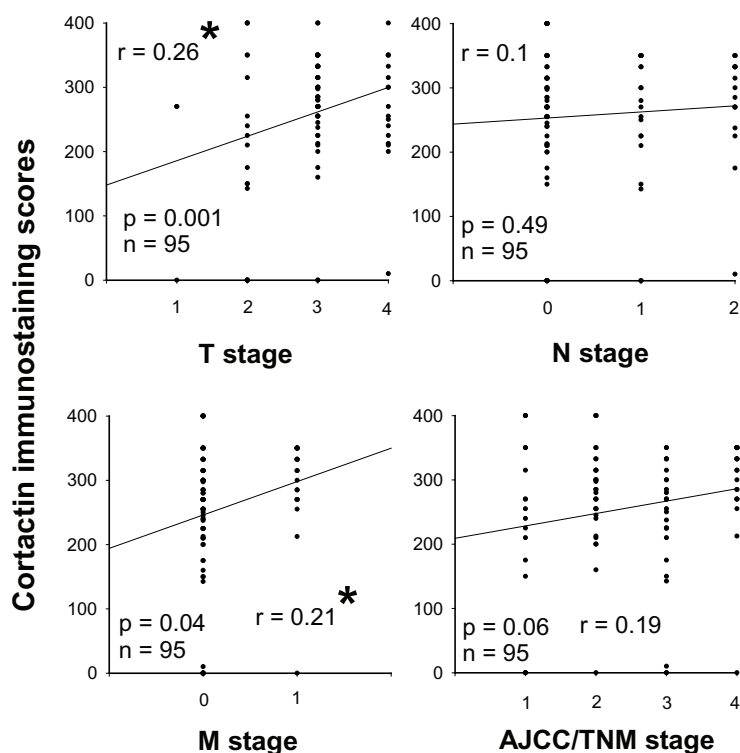
Fig. 3. Correlation of immunostaning scores of cortactin and clinicopathological parameters in colorectal adenocarcinoma.
*Indicates statistical significance of Pearson's product moment correlation ($p < 0.05$).

ability of the chemical signal generated under different environmental conditions [21]. Recent results support the reliability of immunohistochemistry conducted on tissue microarray slides [21]. In our study, the clear cut difference in survivin and cortactin staining between colonic adenocarcinoma tissue and normal glandular epithelia validated the use of tissue microarray slides.

Survivin, an anti-apoptotic factor, is associated with increased tumor aggressiveness and a poorer prognosis in nasopharyngeal, esophageal, liver, pancreatic, colorectal, renal, urinary bladder, hematological, and other malignancies [12,22–28]. However, the mechanism of survivin expression in colorectal adenocarcinomas remains unclear. In our study, higher survivin immunostaining score was observed in colorectal adenocarcinoma but not in tubular adenoma, suggesting an important role of survivin in malignant transformation from colorectal tubular adenoma to adenocarcinoma. The higher survivin immunostaining score in colorectal adenocarcinoma was significantly associated with more advanced T, M, AJCC/TNM stages and lower survival rate.

Previous studies analyzing the prognostic significance of survivin expression were performed without
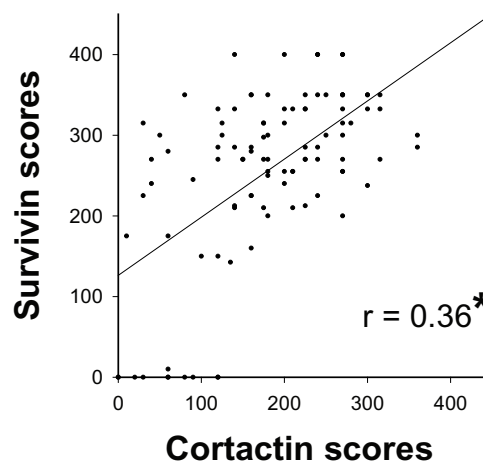

Fig. 4. Correlation of survivin immunostaining scores with cortactin immunostaining scores in colorectal adenocarcinoma.
*Indicates statistical significance of linear regression testing ($p < 0.05$).

taking into account the exact survival time and immunostaining score [40,41]. Ponnelle et al. graded the mean percentage of positive tumor cells from 0 to 4 and the lacking use of tissue microarray predisposed the

Table 4
Cox regression univariate survival analysis

Variable	Hazard ratio (95% CI)	Worse prognosis	P
Survivin score: high vs low	2.25(1.54–4.36)	High score	0.02*
Cortactin score: high vs low	1.02(0.85–1.21)	None	0.52
Age	1.00(0.94–1.12)	None	0.86
Sex	0.54(0.21–0.75)	Male	0.001*
Tumor differentiation:		Poor differentiation	0.004*
moderate vs well	0.78(0.42–2.41)		0.84
Poor vs well	3.54(1.32–7.65)		0.008*
poor vs moderate	4.12(1.78–8.65)		0.001*
T stage: (size)		Large size	0.002*
T2 vs T1	1.35(0.45–6.21)		0.72
T3 vs T1	1.84(0.54–7.65)		0.41
T4 vs T1	10.32(4.12–38.25)		0.005*
T4 vs T2	5.12(2.27–7.86)		0.008*
T4 vs T3	4.65(1.54–8.68)		0.02*
N stage (lymph node)		Positive	0.001*
N1 vs N0	2.87(1.52–5.78)		0.002*
N2 vs N0	5.42(1.65–9.21)		0.005*
N2 vs N1	1.80(0.50–6.54)		0.322
M stage (metastasis)	3.21(1.21–6.27)	Positive	0.021*
TNM stage:			0.042*
II vs I	1.42(0.45–6.21)		0.65
III vs I	2.87(0.77–8.75)		0.21
IV vs I	4.12(0.84–9.78)		0.08
IV vs II	3.45(1.87–6.58)		0.021*
IV vs III	1.45(0.51–3.87)		0.55

Survivin score: low (score < 180) vs high (score ⩾ 180). Cortactin score: low (score < 275) vs high (score ⩾ 275).
*Indicates significant difference in univariate survival analysis ($p < 0.05$).

Table 5
Cox regression multivariate survival analysis (stepwise selection)

Variable	Hazard ratio (95% CI)	Worse prognosis	P
Survivin score: low vs high	1.6 (1.02–2.51)	High score	0.04*
Sex	0.6 (0.37–0.94)	Male	0.02*
Tumor differentiation	3.2 (1.62–6.39)	Poor differentiation	0.001*
T stage (size)	4.9 (1.08–21.72)	Large size	0.04*
N stage (lymph node)	1.9 (1.07–3.24)	Positive	0.03*
M stage (metastasis)	2.5 (1.43–4.30)	Positive	0.001*

Survivin score: low (score < 180) vs high (score ⩾ 180). Cortactin score: low (score < 275) vs high (score ⩾ 275).
*Indicates significant difference in multivariate survival analysis ($p < 0.05$).

variability of the chemical signal generated under different environmental conditions [40]. Lin et al. defined cases with less than 10% positively stained cells were as negative, cases with 10 to 29% positively stained cells were as " + ", 30 to 59% as " ++", and 60% or more than 60% as " + + + " [41]. Sarela et al. described the mean percentage of positive tumor cells as five categories: (1) 0, < 5%; (2) 1, 5% to 25%; (3) 2, 25% to 50%; (4) 3, 50% to 75%; and (5) 4, > 75%. The intensity of survivin immunostaining was scored as (1) weak, 1+; (2) moderate, 2+; and (3) intense, 3+ [42]. The percentage of positive tumor cells and staining intensity were multiplied to produce a weight-

ed score for each case which was similar to our study. However, this study just involved the stage II colorectal carcinomas and did not use of tissue microarray [42]. Our study analyzed the immunostaining scores in tissue microarray and the use of immunostaining scores also made our study more objective and of less bias than the previous studies.

Our current study was designed using tissue microarray. One tissue core per patient was used for construction of tissue microarray. We have verified the immunohistochemistry results in whole sections of 10 cases with colorectum adenocarcinoma and the results are consistent with the immunohistochemistry findings
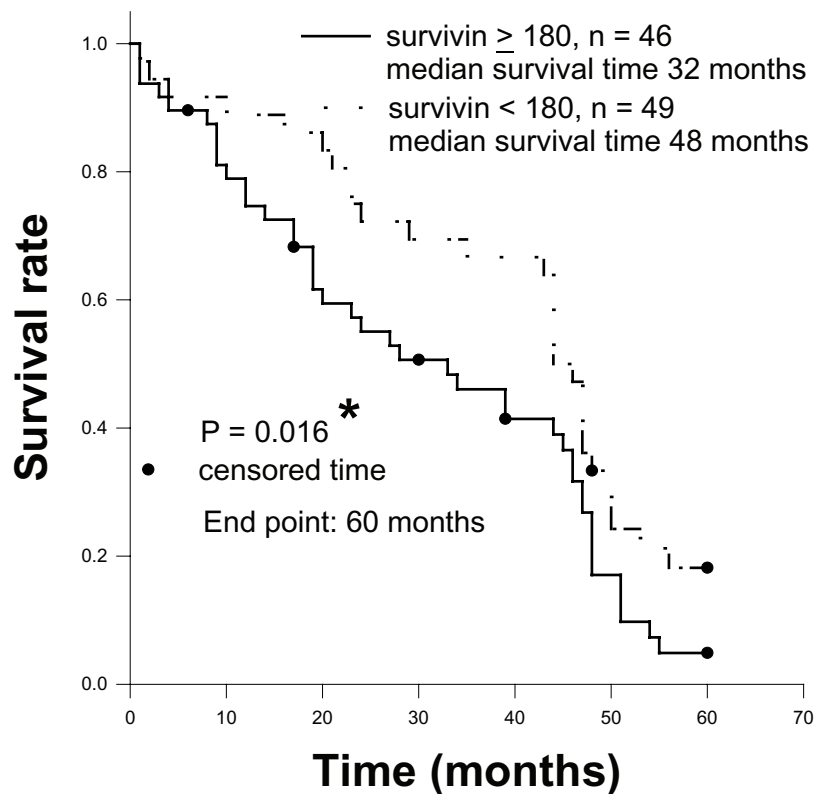
Fig. 5. Overall survival of 95 patients with colorectal adenocarcinoma. Higher survivin immunostaining score ($\geqslant$ 180) was significantly associated with poorer survival rate. Survival rate was analyzed using Kaplan-Meier method.

in tissue microarray slide. The potential limitation of tissue microarray is the correct representation of each tumor with the level of heterogeneity. However, a study has demonstrated that when the number of cases is increase to more than 54 cases in tissue microarray preparation, the probability that results from one core would correctly represent the whole section was more than 91% [38]. Tissue microarray technique enables simultaneous histological and immunohistochemical analysis of a collection of tumor samples [39]. The advantage of the tissue microarray technique is that it is carried out under the same conditions and all samples are evaluated simultaneously on a single tissue microarray slide [39].

Cortactin regulates the actin cytoskeleton through its involvement in cell motility, adhesion, polarization, contraction, etc. [29,30]. The activation of actin-related (Arp) 2/3 protein complex and neuronal Wiscott-Aldrich syndrome protein (N-Wasp) by cortactin regulates actin polymerization and promotes cellular motility. Cortactin is a p80/p85 multidomain actin filament-binding protein [31]. Human cortactin maps to chromosome 11q13 [32]. Amplification of chromosome

11q13 has been reported in several human carcinomas with increased expression of cortactin [33]. Over-expression of cortactin induces cell motility and migration, inhibits cell-cell adhesion, and accelerates tumor spreading [13]. In addition, the effects of cortactin may be related to expression of E-cadherin and its effects on intercellular adhesion [34–36].

In some *in vitro* studies, cortactin over-expression induced tumor invasion and metastasis in esophageal and head/neck squamous cell carcinomas [32,37]. However, a relationship between cortactin over-expression and tumor progression and metastasis has not been established in colorectal adenocarcinomas. Our current results demonstrate that the expression of cortactin is higher in the colorectal adenocarcinoma and tubular adenoma than in the normal colorectal epithelia, and that higher cortactin immunostaining score is associated with more advanced stages (T, M) (Table 3, Fig. 3).

In conclusion, higher survivin immunostaining score is associated with more advanced T, M, and AJCC/TNM stages, and shorter survival time in colorectal adenocarcinoma. Similarly, we found a correlation between higher cortactin immunostaining score
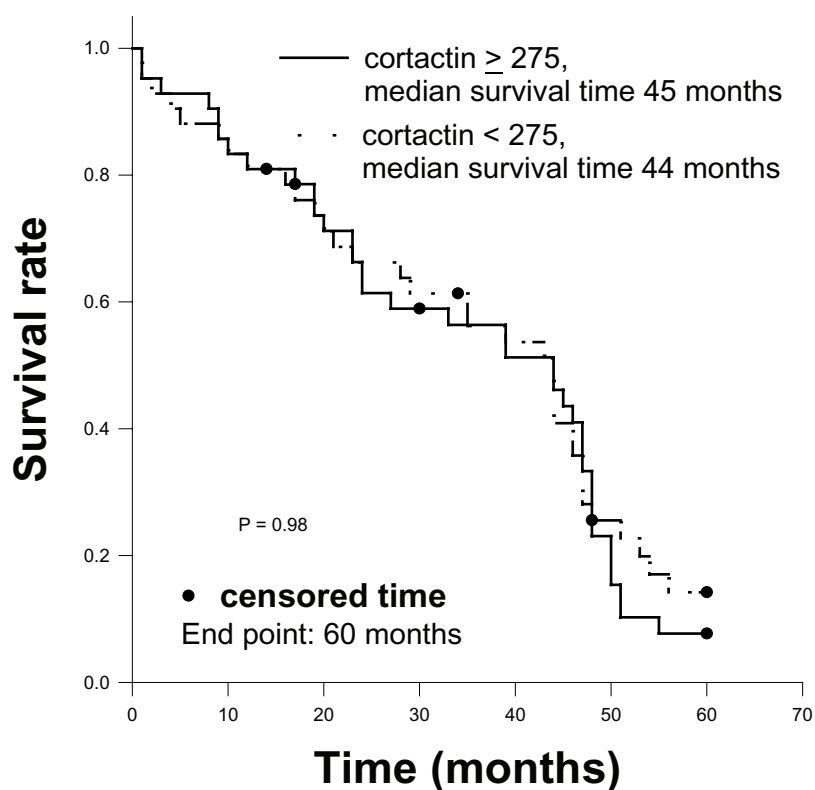
Fig. 6. Overall survival of 95 patients with colorectal adenocarcinoma. Higher cortactin immunostaining score ($\geqslant 275$) was not significantly associated with poorer survival rate. Survival rates were analyzed using Kaplan-Meier method.

and T and M stages in colorectal adenocarcinoma. Although their role in tumor progression is still unknown, our results suggest that survivin and cortactin are appropriate biomarkers for predicting clinical outcomes in colorectal adenocarcinoma.

Acknowledgments

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